

COMPARATIVE STUDIES IN THE DIAGNOSIS OF NEWCASTLE
DISEASE, IN EXPERIMENTALLY INFECTED CHICKENS,
USING EGG INOCULATION, FLUORESCENT ANTIBODY
AND HISTOPATHOLOGIC TECHNIQUES

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Summary

White Leghorn chickens (5, 7, and 9 weeks old) were intramuscularly infected with various dilutions of Salsbury Strain NDV. Chickens with severe clinical signs were euthanatized by exsanguination and necropsied. Lungs, trachea, proventriculus, brain, spleen, and bone marrow were collected. Each tissue was processed and examined for NDV infection by each of a standard technique of embryo inoculation (EI), fluorescent antibody (FA), and microscopic examination.

Higher doses of the virus produced greater clinical evidence of infection by each test. Positive results from 275 tissues each were obtained from 213, 204, and 195 samples by EI, microscopic examination and FAT respectively. Results of EI and FAT showed 90 per cent agreement. Lung, proventriculus, trachea, and spleen were the most frequently infected tissues. Direct FAT was the simplest, fastest and most specific test for routine diagnosis.

Introduction

Virulent Newcastle disease (ND) is probably the most important poultry epizootic in the world. Virulent ND has become a serious threat to the intensive poultry industries of developed countries as well as the backyard poultry flocks in developing countries (28, 67). In tropical and subtropical countries where velogenic viscerotropic ND is endemic,

whole flocks have been destroyed by high mortalities and serious nervous involvement (28, 44, 56). The mild form of ND, in temperate regions, caused severe economic losses because of loss of egg production, poor egg qualities, severe mortalities in young flocks and resulting nervous deformities (4, 9, 52, 58, 77).

The history, clinical picture, gross and microscopic lesions of ND resembled those of other poultry diseases such as nervous, respiratory or deficiency diseases and poisonings (44). The success of control measures, such as quarantine, vaccinations, slaughter and compensation, depend largely on a rapid diagnostic technique which accurately demonstrates the presence of NDV.

The injection of suspensions of suspected tissues into the allantoic sac of 9-11 day, healthy, embryonating chicken eggs, incubated at 37-38°, has been a standard method for isolating NDV. The embryos were examined for pathologic lesions and the allantoic fluids (AF) tested for hemagglutinating activities (HA). Isolation of NDV was confirmed when NDV specific antiserum inhibited the HA of the allantoic fluids (2, 14, 26, 42, 44, 72, 86). The inoculation procedure has been a reliable diagnostic method for isolating NDV but may require 2-17 days for completion, depending on the availability of fertile eggs and the strain of the virus involved (72). Histopathologic examination of tissues of birds with suggestive histories of ND has been used to support clinical (or other) diagnosis of ND in endemic areas (44, 56). This diagnostic technique provided only supportive evidence of the presence of NDV and came too late for any prompt and effective initiation of control measures.

The fluorescent antibody technique, (FAT), first developed in 1941 (32) was used to visualize the presence of diverse antigenic

substances in tissue sections and smears. FAT has been used extensively in the detection of bacteria, viruses, helminths, hormones, enzymes, and antibodies (8, 21, 22, 31, 33, 34, 49, 63, 64, 89). The application of FAT to detect the presence of NDV antigens in tissue sections was developed in studying the pathogenesis of the virus (7, 24, 25, 26, 35, 63, 78). FAT had the advantage of being a rapid and sensitive test once the procedures were standardized with adequate controls.

In the following experiments, embryo inoculation, fluorescent antibody and histopathologic techniques were compared for the diagnosis of ND, in selected tissues of 5, 7 and 9 week-old, artificially infected and uninfected control chickens.

Review of Literature

Newcastle disease is an infectious, highly contagious and destructive disease of poultry caused by a filterable paramyxovirus.

Newcastle disease occurs primarily in chickens and turkeys (12, 13) while ducks and pigeons are susceptible to a lesser extent (75). Only a few avian species are resistant to the disease but carnivorous birds appear to be entirely resistant to Newcastle disease (12).

Some mammalian species, including man, may have a generalized or localized infection, occasionally with prominent clinical signs, after exposure to Newcastle disease virus (NDV) (12, 36, 38).

Newcastle disease was first recognized as a specific disease of fowls in 1926 by Kraneveld in Indonesia (88). Kraneveld described the disease as an acute lethal infection of chickens of all ages with hemorrhagic lesions of the gastrointestinal tract and in which mortality was almost 100%. In 1927 (37) Doyle described an outbreak of poultry

disease in Newcastle-on-Tyne in which a virus was isolated and shown to be different from any previously known to infect poultry. This was called Newcastle disease and was similar to the disease reported by Kraneveld--including hemorrhages of the gastrointestinal tract, especially in the proventriculus.

Nine years later, a new disease, subsequently proved to be Newcastle disease appeared insiduously in California and later spread throughout the United States (4, 5). The American form of the disease was clinically different and milder than its Indonesian or English counterparts. The mild American form was characterized by low mortality in adult birds but high mortality in young birds. Respiratory signs were more evident and were usually followed by nervous signs such as bilateral twitching, paresis of the legs and torticollis. There was no characteristic lesions in the gastrointestinal tract (53). Nervous lesions, in both the American and Asiatic or Doyle's forms of the disease, were characterized by various degenerative changes of the neurons (1, 53, 54, 56, 88).

Each of the several strains of NDV produces typical clinical signs and lesions characteristic for the viral line. The disease may be subclinical with no mortality (6, 69, 84). It may be mild and chronic, with low mortality in adult birds but high mortality in young chicks causing considerable production loss and such sequellae as respiratory disorders and nervous complications (4, 52).

In most tropical and subtropical regions the peracute virulent forms, the so-called Asiatic forms, are encountered, which cause high mortality with characteristic hemorrhagic lesions in the gastrointestinal tract and generalized petechiae on serosal surfaces. Survivors may

exhibit typical nervous and respiratory signs of the mild American form of the disease. The Asiatic form of Newcastle is caused by the highly virulent or velogenic strains of NDV. A moderate form of the disease, in which the signs, lesions and mortality lie between those of the mild American and the peracute virulent Asiatic forms, is caused by the mesogenic virus strains. The moderate form is found in both temperate and tropical regions of the world. The NDV strains causing subclinical and mild (American) forms of the disease are termed lentogenic. Most Newcastle disease vaccines are made with the lentogenic strains of the virus (45, 46, 56, 60).

Embryo Inoculation

The high susceptibility of the developing chicken embryo to infection by NDV has made it a suitable sterile medium for isolation of the virus from suspensions of suspected tissues (2, 19, 44). All strains of NDV caused infection of the chicken embryo and the majority led to embryo death (2, 14, 19, 44). Various routes of inoculation of the developing embryo, such as the chorioallantoic membrane (CAM), yolk sac, intravenous (IV), and the allantoic sac (AS) have been used. The route of inoculation influenced the resulting virus titer. The following routes--CAM (19), IV (47) and AS (2, 47, 54) were very productive. The AS route was the easiest and most frequently used method of inoculating chicken embryos.

The age of the embryo had a significant effect on its resistance to NDV infection. The younger the embryo, the more susceptible it was to infection (10, 47, 83). Nine to 12-day embryonating eggs were usually employed for viral isolation, 10-day-old embryos being the standard. At this age, embryonic tissues were sufficiently developed for easy

manipulation and passive antibodies, which may be present in the yolk, had not yet entered the circulation (15, 44, 72). Tissue distribution and concentration of the virus were dependent on the strain as well as the dosage of the inoculum. In general, higher dosages of the inoculum produced higher embryonic tissue titers and, per given dilution of inoculum, the more virulent strains produced higher titers than the less virulent strains (44, 47). Incubation temperature above 37°C promoted a rapid fatal infection (86).

The pathology of NDV in developing embryos has been described by several workers (2, 10, 14, 19, 47, 54, 71, 83, 92). NDV caused general retardation in growth of the embryo and in very young embryos malformations and organ defects were demonstrated. In older embryos, varying degrees and amounts of hemorrhagic lesions, necrotic foci, edematous swellings and cloudiness of embryonic and extra-embryonic tissues have been observed. Microscopically, NDV caused inflammatory cell accumulations, edema and hemorrhage, in the mesoderm and ectodermal cell proliferation accompanied by or associated with prominent vacuolation of epithelial cells and the destruction of microvilli. The virus was located deeply or superficially within affected cells.

Hemagglutination Test

The hemagglutination test was shown to be applicable to NDV identification (18, 62) in a similar manner to that characteristic of influenza viruses (50, 66). The test was later elaborated and standardized (14, 16, 44, 47, 72) so that the principal requirements for the tests were 1) fresh chicken erythrocytes, 2) a diluent containing an electrolyte (0.85% NaCl) and 3) the presence of 10^5 - 10^{10} virus particles per ml in the suspension of infected tissue (or AF). The mixture was

incubated at room temperature, with frequent observation, for 30-60 minutes or in a refrigerator for longer periods.

Variations, in the results, occurred with prolonged storage of erythrocytes (over 5 days); non-uniformity of strains, virus modification, or attenuation, route of embryo inoculation, incubation temperature, differences in erythrocytes of individual chickens or as a result of contamination with an erythrocyte agglutination principle present in the yolk sac (16).

Histopathology

The pathologic expression of ND infection in chickens has been reported to be associated with the strain of virus (7, 27, 44, 54, 55, 56, 85) and, in part, by the route of administration (1, 7, 54, 59), the age of the chicken (44), and by environmental factors such as temperature (86). The pathogenicity of individual strains was also modified by the breed of the host (30) and the host's acquired immunity (44, 54, 60), while development of respiratory disease was often associated with natural routes of infection (respiratory and intestinal epithelium) (7), intramuscular, intravenous, and intracerebral routes usually enhanced neurovirulence of the virus (44).

Lesions of ND generally were in the following categories:

(a) lesions associated with the vascular system - generalized hyperemia, edema and hemorrhage in interstitial tissues (b) foci of necrosis in parenchymal organs such as the liver, lungs, kidneys, brain and heart, (c) diffuse necrosis of reticuloendothelial cells including macrophages, endothelial cells and lymphocytes of the spleen, bursa of Fabricius, thymus and intestinal lymphoid cells and (d) diffuse and/or focal infiltration of mononuclear cells, chiefly lymphocytes, of affected organs and tissues.

Trachea. In a report of experimental infection of ND (53), mild, irregular, edematous swelling with localized deep desquamation of mucosa and congestion of subepithelial vessels were observed in the trachea. In aerosol vaccinated birds (7), marked involvement of the tracheal epithelium with loss of cilia, engorgement of capillaries and extensive mononuclear infiltration were seen on 3rd and 4th days p.i. and these lesions regressed from the 6th through the 10th day when cilia reappeared on epithelial surfaces. A virulent strain (NDV-Largo) caused only marked focal destruction of epithelia of tracheas, sinuses, and nasal cavities in another report (27).

Lungs. In severely affected lungs (53) pulmonary consolidation with heterophil and monocytic infiltration were reported. In a study of the comparative pathology of fowl plague and ND (54) lung lesions produced by ND were primarily proliferative and secondarily exudative. There was hypertrophy and hyperplasia of alveolar wall cells and endothelial lining of capillaries resulting in partial or complete obliteration of alveolar spaces with subsequent consolidation and interstitial pneumonia. Cellular serous accumulations were seen in the peribronchial lumen with extension into the lumen of tertiary bronchi. Cells in the exudate included desquamated pulmonary cells, macrophages and disintegrating heterophils and erythrocytes. One virulent strain, NDV-219, caused limited to severe hyperemia and moderate edema of the parabronchi while another virulent strain, NDV-Largo, caused more extensive hemorrhagic lesions including erythrophagocytosis in the lungs (27).

Proventriculus. Hemorrhagic lesions of the mucosa and hemorrhagic, necrotic involvement of lymphoid patches and follicles of proventriculus and intestines were more prominent and constant with

European strains of NDV (54). These hemorrhagic necrotic lesions were characterized grossly as diphtheritic ulcers (73).

Spleen. The incidence of necrotic foci in the spleen, with hyaline degeneration of splenic follicles and erythrophagia varied from frequent to rare (54). Cheville et al. (27) observed agglutination and lysis of erythrocytes in splenic red pulp as well as erythrophagocytosis by macrophages lining the sinuses and varying amounts of focal necrosis of reticuloendothelial cells. In another study (25) dilation of sinusoids, swelling of endothelial cells, stasis and destruction of erythrocytes and extensive erythrophagocytosis were observed in the red pulp. Swelling of arteriolar and reticular cell sheaths, hydropic degeneration of endothelial cells, fibrinoid necrosis of medial myocytes and necrosis of adventitial cells were also observed.

Brain. Lesions of non-purulent encephalitis consisting of diffuse lymphocytic infiltration, capillary hemorrhages, myelin degeneration, endotheliosis, meningitis, cytoplasmic disintegration of the neurons, glia foci, and perivascular cuffing have been observed in medulla oblongata, thalamus and cerebellum (53, 54). Primary neuronal injury, diffuse lymphocytic infiltration and perivascular cuffing distributed throughout the brain were described (55). Generalized lymphocytic infiltration, extensive hyperemia and some degeneration of ganglion cells were also observed in field cases of the disease (1). The predominant changes found in chickens infected intramuscularly (88) were extensive hyperemia of the brain accompanied by endothelial cell proliferation. Neuronal degeneration ranged from mild chromatolysis to frank necrosis. These lesions were observed primarily in the reticular,

vestibular and cerebellar nuclei and in Purkinje cells. In a group of infected chickens, extensive gliosis, neuronal degeneration and endothelial cell proliferation were observed in brains in which lymphoid infiltrates were not observed (25). In a separate study (27) lesions were seen throughout the brain but were more common in the Purkinje cell layer.

The severity, extent and tissue distribution of histologic lesions caused by NDV was enhanced or limited principally by the strain of virus and, in part, by host factors and by factors of dose, route of administration and environment.

Fluorescent Antibody Technique (FAT)

Fluorescent Antibody Technique utilized specific antiserum conjugated with a fluorescent protein dye to visualize antigenic substances in tissues. The technique was developed in 1942 (32) and was later improved and detailed (33, 34).

The technique has been used in various fields of experimental pathology, immunologic research, cytologic investigations and diagnostic microbiology. Among the great variety of antigenic substances that have been studied with FAT are bacteria, viruses, helminths, enzymes, hormones, immunoglobulins (7, 8, 22, 25, 26, 27, 31, 41, 48, 64, 70, 74, 78). The application of direct FAT in the detection of NDV antigens has been largely limited to the studies of pathogenesis and viral multiplication in adult and embryonic avian tissues and in primary avian and mammalian tissue culture cells.

Fluorescent antibodies have been used to study the multiplication of NDV in tissue culture cells (17, 78, 80, 91), and the location of NDV in chorioallantois of infected chicken embryos (26). The distribution

and pathogenesis of NDV in organs of infected chickens has been demonstrated with the use of FAT (7, 20, 25, 26, 27, 35, 74).

The equipment, materials and procedures involved in preparing the specific antiserum, conjugate, removal of non-specific staining, staining and microscopic examination of stained tissues have been critically described and reviewed (3, 8, 11, 23, 24, 29, 39, 40, 43, 51, 57, 65, 68, 76, 79, 82, 87, 93).

The introduction of commercial, polyvalent NDV specific, FITC-conjugated immunoglobulins (Salsbury Laboratories) and the availability of microscopes equipped with high quality optics for fluorescence microscopy have eliminated many of the problems associated with antibody preparation, conjugation, non-specific staining and have improved the readability of the stained tissues. These factors together with the rapidity, specificity and simplicity of the test encouraged the application of the direct FAT for routine diagnosis of ND in poultry.

Materials and Methods

Chickens - Male, unvaccinated, day-old, White Leghorn chicks were obtained from a commercial hatchery. The parent flocks had no history of Newcastle disease (ND), influenza or mycoplasma infection.

Virus - A stock NDV (Salsbury Strain)* in the form of allantoic fluid (AF) was used.

Chicken embryos - Fresh fertile eggs for incubation were obtained from a commercial hatchery. Parent flocks were not vaccinated for ND.

Conjugate - Polyvalent NDV specific fluorescent antibodies (List No. 7278), in the form of desicated fluorescein isothiocyanate

*Supplied by Dr. A. L. Burroughs, Department of Infectious Diseases, K.S.U.

(FITC) conjugated with anti-NDV specific chicken immunoglobulins were obtained from Salsbury Laboratories, Charles City, Iowa.

Experimental Infection

Chicks were raised in brooder-grower cages until at 5, 7, and 9-weeks old, groups of 21, 15 and 20, respectively, were transferred into another room and placed in a Germ-Free Plastic Isolator* (6MP-TMP) fitted with inlet and outlet filters, exhaust air incinerator unit and operated under negative pressure.

Group I

Twenty, 5-week-old chickens were placed in the isolator and divided into 4 groups of 5 birds each. One tenth ml of serial dilutions (10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8}) of the stock virus (AF), in sterile phosphate-buffered saline (PBS) with penicillin and streptomycin in concentrations of 250 units and 250 ug/ml respectively, was administered intramuscularly in the breast muscles. Birds were observed for clinical signs, morning and evening, for 12 days post-inoculation and the observations recorded. Chickens that became comatose were euthanatized by exsanguination, necropsied and the gross pathologic lesions noted.

Controls

Five, 5-week-old uninfected control birds were inoculated intramuscularly with 0.1 ml of sterile PBS containing penicillin and streptomycin--concentrations of 250 units and 250 ug/ml, respectively. Two control chickens each were euthanatized on days 5 and 12 and the remaining chicken on day 30 post-inoculation.

*Germ-Free Lab., Inc., Miami, Florida.

Group II

Fifteen, 7-week-old chickens were placed in the isolator and each was inoculated intramuscularly with 0.1 ml of the stock virus having a calculated infectivity titer of $100 \times 10^{8.3}$ ELD₅₀/ml. Birds were observed for clinical signs, morning and evening, and the results recorded. Chickens with the most severe clinical signs were euthanatized and necropsied on days 2, 4, 5, 6, 8, 10 and 12.

Controls

Three, 7-week-old uninfected control birds were treated in the same manner as Group I controls except that they were euthanatized, one each, on days 2, 6 and 12 post-inoculation.

Group III Chickens

Twenty, 9-week-old chickens were treated exactly as Group II infected chickens.

Controls

Five 9-week-old chickens treated in the same manner as Group I controls. Two chickens each were euthanatized on days 2 and 6 and the remaining chicken on day 12 post-inoculation.

Tissue Collection

At necropsy, brain, trachea, lung, proventriculus and spleen were collected separately and cut into 3 approximately equal parts--one part each for embryo inoculation (EI), histopathology and fluorescent antibody technique (FAT). Specimens for EI and FAT, including whole femurs, were wrapped individually in aluminum foil, identified, placed in sterile, plastic bags and stored at -20°C until examined.

Tissue specimens for histopathologic examination were placed in 20 volumes of buffered, neutral 10% formalin (BNF) until they were embedded, sectioned 6 microns thick, and stained with hematoxylin and eosin by a standard (short cycle) technique.

All tissue sections were examined microscopically and were considered positive if inflammatory lesions were observed.

Embryo Inoculation

Fertile eggs were incubated at 37.5°C and candled morning and evening.

Tissues for embryo inoculation were ground in sterile porcelain mortars with pestles. One to 2 ml of PBS containing 500 units of penicillin and 500 ug of streptomycin/ml were added and the emulsion centrifuged. The supernatant was drawn into tuberculin syringe having a 22G, one-inch needle. Ten-day embryonating eggs were disinfected over the air sacs by swabbing with 2% iodine in 70% ethyl alcohol. A hole was made in the shell about 1 cm above the limiting edge of the air cell. The needle was inserted 0.1 ml of the inoculum deposited in the allantoic sac. The hole was plugged with heated paraffin wax-vaselin (4:1) mixture. Five embryos were inoculated with each tissue extract. Inoculated embryos were returned to the incubator and candled morning and evening for five days. Embryo death and/or the presence of HA in the AF was considered positive evidence of NDV isolation.

Controls

Embryos inoculated with tissue extracts from uninfected control chickens were used as controls.

HA Titration

Blood of control chickens, collected in 9:1 ratio of blood to 4% sodium citrate, was mixed 50/50 with PBS, centrifuged and decanted. The sediment was suspended in 50 volumes of PBS, then centrifuged and decanted. The washed packed erythrocytes were stored as 50% suspension at 4°C for not more than 5 days.

Serial doubling dilutions of pooled allantoic fluids were made with manual microdiluters in clean, disposable, white plastic plates containing 8 x 12 rows of wells. Equal amounts of 0.5% washed erythrocytes were added to each well.

Controls

Wells containing allantoic fluid dilutions of control embryos plus erythrocytes and PBS plus erythrocytes were used. The plates were incubated at room temperature for 45 minutes and read. The procedure is outlined in Table I below.

Table 1. Titration of Hemagglutinating Activity Quantity (ml)

Well	PBS	Virus	Transferred	0.5% rbc	Dilution of Virus	Sample Result
1	0.05	0.05	0.05	0.05	2	+
2					4	+
3					8	+
4					16	+
5					32	+
6					64	+
7					128	+
8					256	+
9					512	-
10			*		1024	-

*.05 discarded

+ agglutination

- no agglutination

Fluorescent Antibody Technique

Frozen tissues were cut, 6 μ thick, with microtome cryostat and mounted on slides. Bone marrow from the neck of the femur was smeared on slides. Slides were air dried at room temperature for 10 minutes and fixed for 20 minutes in anhydrous acetone at 4°C. Slides were air dried for another 10 minutes. The desicated conjugate* was reconstituted with 1 ml of sterile distilled water, rotated until fully dissolved and diluted (1:10) with PBS(pH 7.4). Diluted conjugate (0.1 ml) was applied to the tissue sections and smears. The slides were placed in a wet chamber and incubated for 30 minutes at room temperature. Excess conjugate was removed and the slides were washed with gentle agitation for 10 minutes in PBS and the washing repeated in fresh PBS for another 10 minutes. The slides were rinsed finally in sterile distilled water and gently blotted dry. A small amount of FA mounting fluid (buffered glycerin, consisting of 9 parts glycerin and 1 part PBS) was added to the center of each stained area on the slide and mounted with a cover glass. The slides were then examined for tissue associated fluorescence with Leitz Ortholux microscope equipped with appropriate filters and an Osram lamp as the ultraviolet light source.

Controls

Infected allantoic membranes with allantoic fluid HA titers of 1024 were used as positive controls. Uninfected allantoic membranes with allantoic fluid HA titers of 0 were used as negative controls.

Autofluorescence

In preliminary tests, tissue sections of uninfected control

*Salsbury Laboratories, Charles City, Iowa.

chickens were stained and examined for autofluorescence. Increased dilutions of the conjugate (1:10) above the recommended (1:5-8) by the suppliers reduced non-specific fluorescence.

50% Embryo Lethal Dose Calculation

The method of Reed and Muench (81) was used for calculating ELD₅₀. See Table 2.

Decimal dilutions of stock virus were injected into allantoic sacs of 10-day embryonating chicken eggs. Ten embryos were injected (.01 ml each) per dilution. All embryos were candled twice daily for 7 days. Embryos that died after the first 24 hours and live embryos that had (AF) HA titers on the 7th day were counted as dead. Non-specific deaths were not counted.

Table 2. Estimated ELD₅₀

Log ₁₀ of Dilution	Death Ratio	<u>Individual Results</u>		<u>Accumulated Results</u>			% Mortality
		Alive	Dead	Alive	Dead	Total	
4	10/10	0	10	0	38	38	100
5	10/10	0	10	0	28	28	100
6	7/10	3	7	3	18	21	85.7
7	6/10	4	6	7 ^b	11 ^a	18	61.1
8	4/10	6	4	13 ^d	5 ^c	18	27.7
9	1/10	9	1	22	1	23	4.5

Using the formula of proportionate distance = PD (Reed & Muench (81)

$$PD = \text{Fractional LD}_{50}$$

$$= \frac{(a - b)(C + d)}{2(ad - bc)}$$

$$= \frac{(11 - 7)(5 + 13)}{2[11 \times 13 - 7 \times 5]}$$

$$= \frac{4 \times 18}{2(143 - 35)} = \frac{72}{2 \times 108}$$

$$= \frac{72}{216} \quad \begin{array}{r} 1.85733 \\ -2.33445 \\ \hline -1.52288 \end{array}$$

$$= .3333$$

$$.33$$

Log of dilution = -7.00

+ 0.33 x -1.0 = -0.33

= $\log_{10} LD_{50}$ = -7.33

The stock virus (AF) contained

$$= 10^{7.3} / 0.1 \text{ ml}$$

$$\text{or } 10^{8.3} LD_{50}/\text{ml}$$

Results

Clinical Findings

The clinical course of the disease varied with the titer of the inoculum. Chickens receiving larger doses of the virus had higher mortalities and developed more severe clinical signs. The principal clinical signs included anorexia, weakness, fever, somnolence, paralysis of the legs, prostration and coma followed by death. Chickens inoculated with lower titers and those inoculated with higher titers that survived the 5th and 6th days p.i. showed respiratory signs of gasping with occasional rattling sounds, loss of weight and leg paralysis. Only 2 birds had typical torticollis. A few birds had a greenish-yellow diarrhea on 4th and 6th days p.i.

Gross Pathologic Findings

Chickens that died before the 6th day p.i. were generally in good flesh and had generalized hyperemia of the visceral organs, particularly the intestines, liver, spleen, kidneys and occasionally the cloaca. The lungs were usually congested, edematous and foamy. The serosal surfaces were edematous. Most brains appeared normal but a few birds had slightly congested cerebellums. Chickens that died after the 6th day p.i. were dehydrated, thin, their lungs were gray and foamy and their spleens and Bursae of Fabricius were reduced in size.

Histopathologic Findings

Severe inflammatory reactions of the tracheal mucosa were observed in most of the chickens dying on 4th-6th day p.i. There was diffuse and/or focal lymphocytic infiltration of the epithelial and subepithelial tissues, congestion, edema, focal areas of necrosis, presence of numerous phagocytes and deciliation of epithelial cells. In some cases the tracheal lumen was filled with necrotic cellular debris--chiefly lymphocytes, erythrocytes, phagocytes and desquamated epithelial cells. In many instances the epithelial structure was disorganized.

In birds surviving the 6th day p.i. there was a varying degree of epithelial reorganization, there was less congestion and edema, fewer and more diffuse mononuclear cells, a gradual reappearance of cilia on epithelial surfaces, and there was no exudate in the tracheal lumen.

Histologically the lungs were the most severely affected tissues. There was generalized congestion, perivascular and septal edema, endothelial degeneration, hyperplasia and hypertrophy of parabronchial and bronchial epithelium, diffuse and focal infiltration of mononuclear cells, focal areas of necrosis in the parenchyma filled with phagocytes.

Many bronchi were filled with fibrinous and necrotic cellular debris, mostly macrophages, heterophils, erythrocytes, and desquamated epithelial cells. Birds dying after the 6th day p.i. had necrotic lesions in the lungs.

Proventricular lesions consisted mainly of ulceration and infiltration of glandular tissue by lymphocytes and macrophages, diffuse lymphocytic infiltration of the ganglia and neuronal degeneration.

In the spleen, destructive lesions were more evident from day 4 through day 6 p.i. There was extreme congestion of the red pulp with erythrophagocytosis, endothelial cell degeneration, characterized by vacuolated cytoplasm and pyknotic nuclei, and loss of lymphoid cells. Decreasing numbers of foam cells were observed from 6th to 12 day p.i.

The brains had less tissue reaction than other organs examined. There were diffuse lymphoid infiltrates in the granular and molecular layers of the cerebellar folia, perivascular cuffing and perivascular edema. Some Purkinje cells showed various stages of chromatolysis, dissolution and necrosis.

Histologic lesions associated with ND were observed in 51 lungs, 49 proventriculi, 45 spleens, 44 tracheas and 15 brains of the 55 infected chickens examined. Lesions were not observed in corresponding organs of uninfected control chickens.

FAT

Tissue associated fluorescence, showing the presence of NDV antigens, was particularly intense in parabronchial and bronchial epithelium of the lungs, affected glandular epithelium of the proventriculus, brush border and desquamated cells of the tracheal epithelium and in the cellular debris in exudates in these organs. Less intense

fluorescence was observed in scattered macrophages in the spleen, lung parenchyma and in bone marrow smears. Limited focal fluorescence of a few neurons were seen in only 2 brains. Increased concentrations of the conjugate failed to reveal any definitive fluorescence in the Purkinje cells.

Embryo Inoculation

Death of the embryo and/or the presence of HA in AF of inoculated chicken embryos provided evidence for NDV isolation. Embryos inoculated with tissue extracts of lungs, proventriculi and tracheas had in general higher HA titers and faster Embryo death time (EDT). The few embryos, inoculated with brain extracts, that were positive had the lowest HA titers and the longest EDT. HA titers and EDT for spleen and bone marrow extracts were moderate. Fifty-five lungs, 53 proventriculi, 51 tracheas, 47 spleens, 35 bone marrows, and 7 brains were positive by EI.

The total number of tissues found to be positive for NDV by EI was the highest compared to those by FA and histopathologic techniques. The results are summarized in Table 3.

Table 3. Results of EI direct FAT and histologic examination applied to various tissues of all NDV infected chickens

Diagnostic Tests Applied	Tissues Examined Percent Positive					
	Spleen	Lung	Trachea	Provent.	Brain	Bone Marrow
EI	85.27	100.00	92.73	96.36	12.73	63.64
FAT	72.73	96.36	85.45	96.36	3.64	36.36
Histopath.	81.82	92.75	80.00	89.10	27.27	--

Discussion

Histologic alterations ranged from mild inflammatory reaction consisting of hyperemia and diffuse lymphoid infiltration to edema, diffuse or focal cellular degeneration and necrosis of the reticuloendothelial and parenchymal organs (spleen, lymphoid patches proventriculus, brain, lungs) along with hyperplasia, hypertrophy and desquamation of epithelial cells of the respiratory tract. Lesions were less evident after the 6th day p.i. Where present, lesions tended to be focal and regenerative in character especially in the respiratory tract (7).

There was little evidence of the presence of NDV in the brains as revealed by EI (7) or FAT (2) but histologic lesions (15), mostly mild, were more evident. Six brains with microscopically demonstrable lesions were negative by either EI or FAT tests. These six birds died on 6th to 12th day p.i. Circulating antibodies, which might appear at this time (72, 90) may have had a neutralizing or blocking effect and may partially explain these apparent discrepancies.

Two hundred and seventy-five tissues were examined by all techniques. Positive results were obtained from 213 by EI, 204 by microscopic examination of tissues and 195 by FAT. Since presence of microscopic lesions was merely an indication of a pathologic process and not necessarily diagnostic of ND infection (44) further comparisons between the results of EI and direct FAT were made (Table 4). Minimal histologic lesions, such as mild cases of hyperemia, which might be unimportant in field cases were considered positive along with the more typical lesions described above. See the following photographs.

There was a close, 90 percent, agreement between the results of EI and FAT when all the tissues examined by both techniques were

Table 4. Agreement Between Results of Direct FAT and EI Tests on Tissues of all NDV Infected Chickens

TISSUE	NUMBER OF TISSUES EXAMINED	AGREEMENTS		DISAGREEMENTS	
		+EI and FAT	-EI and FAT	EI+, FAT-	EI-, FAT+
Lung	55	53	0	2	0
Provent.	55	53	2	0	0
Trachea	55	47	4	4	0
Spleen	55	40	8	7	0
Brain	55	2	48	5	0
Bone Marrow	55	20	20	15	0
Total	330	215	82	33	0
		297			
Percent		90.0		10.0	0

+ = positive

- = negative

considered. The disagreement appeared to be due to "false negatives" by FAT. In evaluating the results of EI and FAT, one major advantage of EI over FAT was probably the virus replication in the embryo, particularly in the chorioallantoic membrane (2, 26, 72). If infection was limited to tiny foci, grinding of a tissue specimen for EI permitted even distribution of viral particles in the inoculum from that extract, thus enhancing the possibility of producing infection of the embryo and a positive diagnosis of ND. It was possible that some tissue sections and bone marrow smears used for fluorescent antibody staining did not contain foci of infection and thus were negative although EI was positive. The absence of false positive results by FAT was a good indication of its specificity.

Based on the results of these tests and the strain of virus used, the tissue examined could be ranked from the one with the highest to the one with the least diagnostic value as follows--lung, proventriculus, trachea, spleen, bone marrow and brain. However, in selecting tissues for diagnostic examination from poultry suspected of ND infection, consideration should be given to the differences in tissue tropisms exhibited by different strains of NDV encountered in the field. In general, the selection of lungs, trachea, spleen, brain and proventriculus would cover the principal tissue tropisms--respirotropism, viscerotropism, neutrotropism, enterotropism and pantropism.

In this study, viral isolation from NDV infected chickens by EI took 12-15 days to accomplish and specific HI with NDV specific anti-serum was not performed as would be necessary in suspected field cases. Tissues for histopathologic studies, in spite of their limited diagnostic value averaged 7 days to process. The equipment, chemicals and reagents

essential for EI and histopathologic techniques plus the cumbersome and protracted nature of both tests made them less desirable for prompt and decisive diagnosis of ND.

Direct FAT, on the other hand, took 3-4 hours for large numbers of specimen to be processed and examined. Since NDV specific antibodies were involved in detecting the presence of viral antigens, further tests of specificity were unnecessary. Preliminary tests with positive and negative controls served to standardize the test so that all subsequent tests were rapid, simple and specific. Furthermore, the relative distribution and concentration of NDV could be observed in most affected tissues. For these reasons FAT should be the test of choice for the routine diagnosis of ND infection except where typing or classification of the viral strain is desired. In this case EI would be the technique of choice.

EXPLANATION OF PLATES

Plate I

Figure 1. Lung. Pneumonitis.
H E, X 100

Figure 2. Lung. Inflammatory cellular exudate
in bronchial lumen. Interstitial
edema. H E, X 100

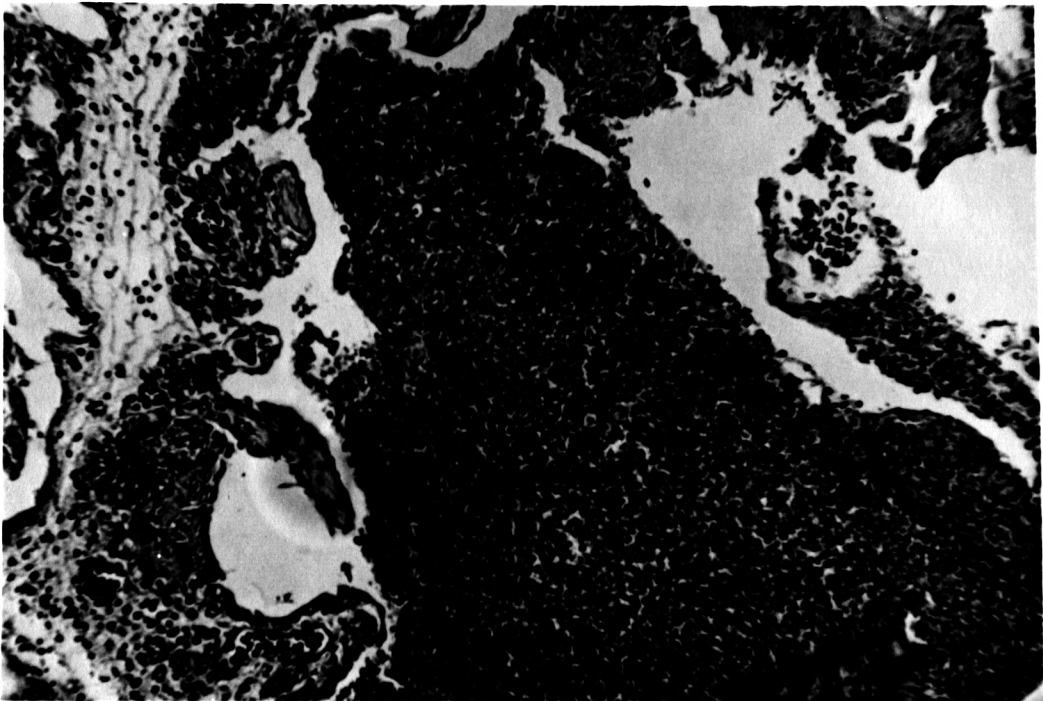
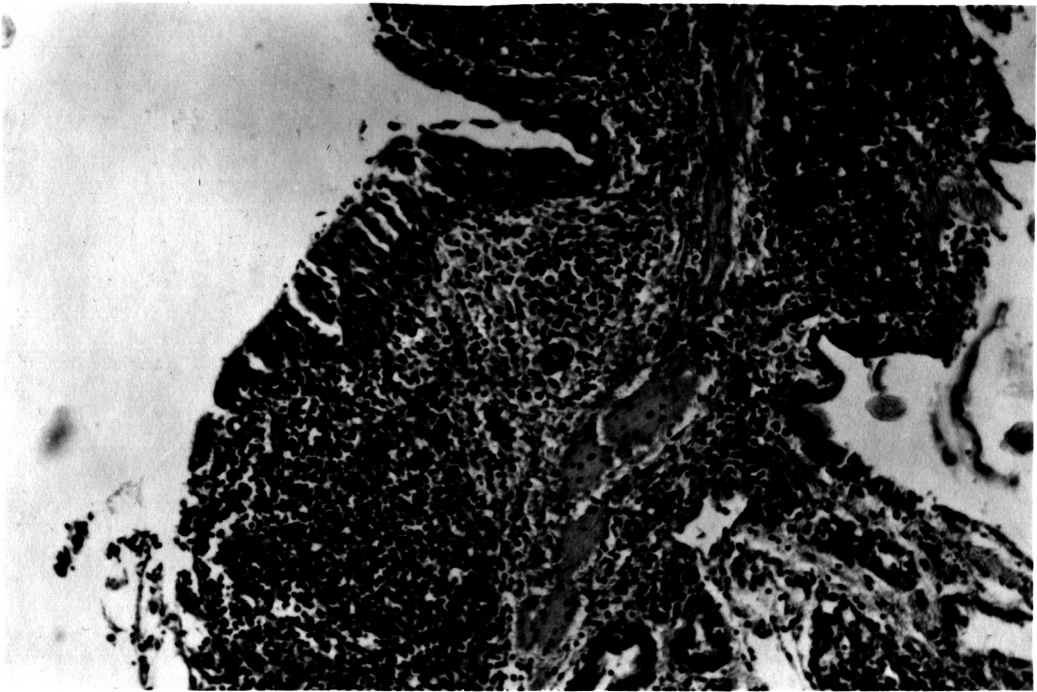


Plate II

Figure 3. Trachea. Subepithelial edema.
Deciliation and congestion of
tracheal epithelium. H E, X 40.

Figure 4. Trachea. Focal area of necrosis in
tracheal epithelium invaded by
lymphocytes and macrophages.
H E, X 100.

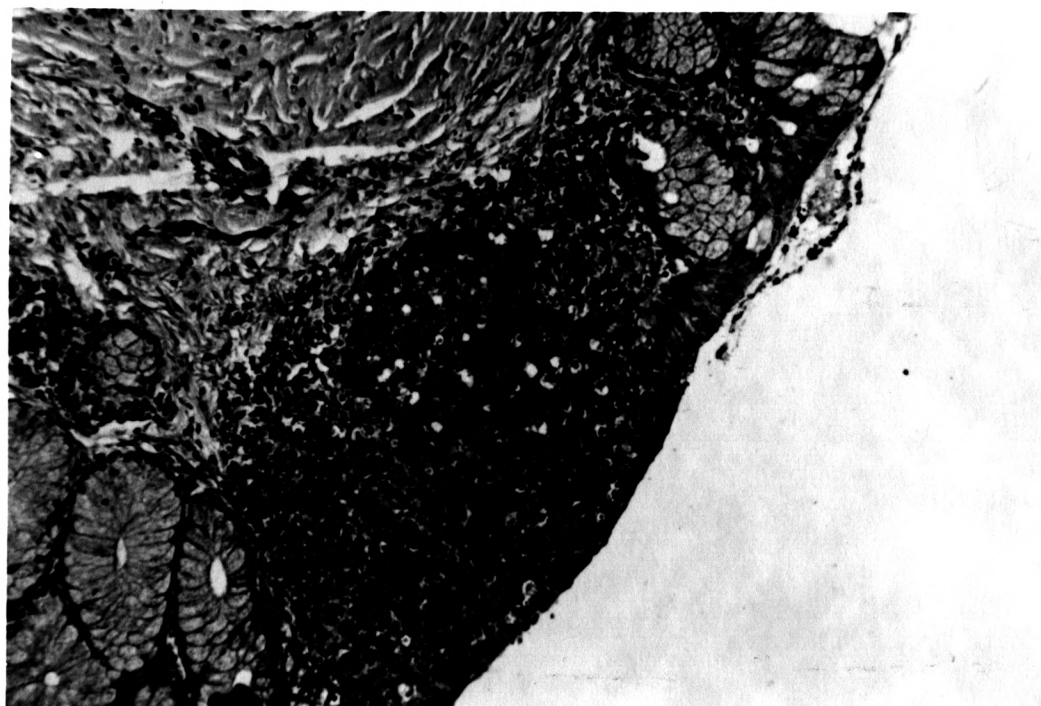
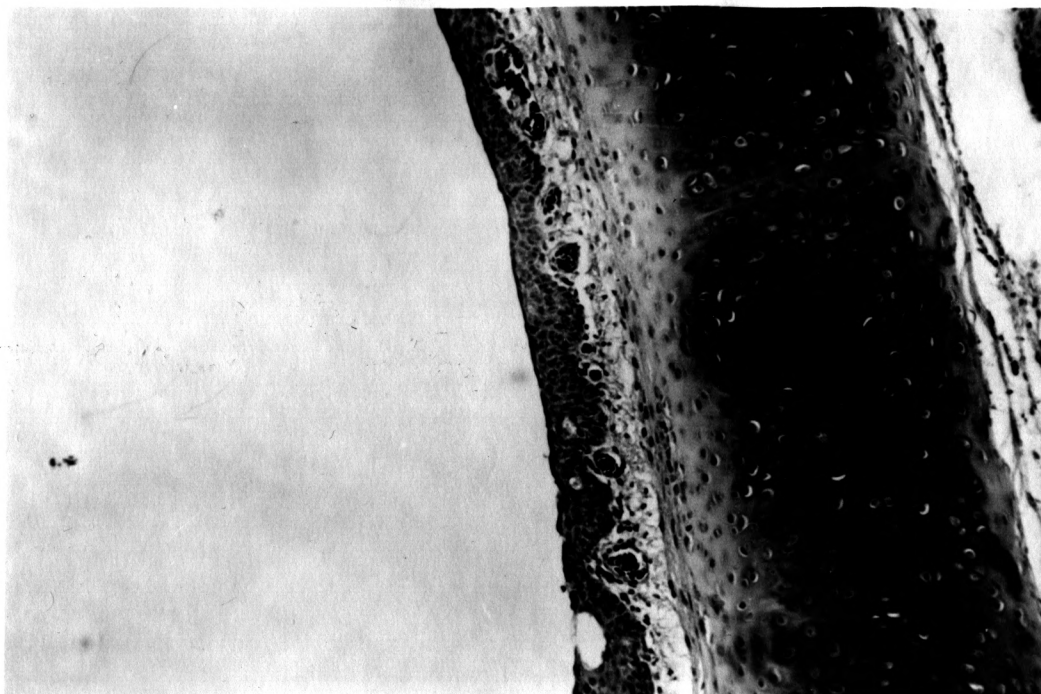


Plate III

Figure 5. Proventriculus. Focal area of ulceration. H E, X 100.

Figure 6. Proventriculus. Focal area of ulceration H E, X 100.

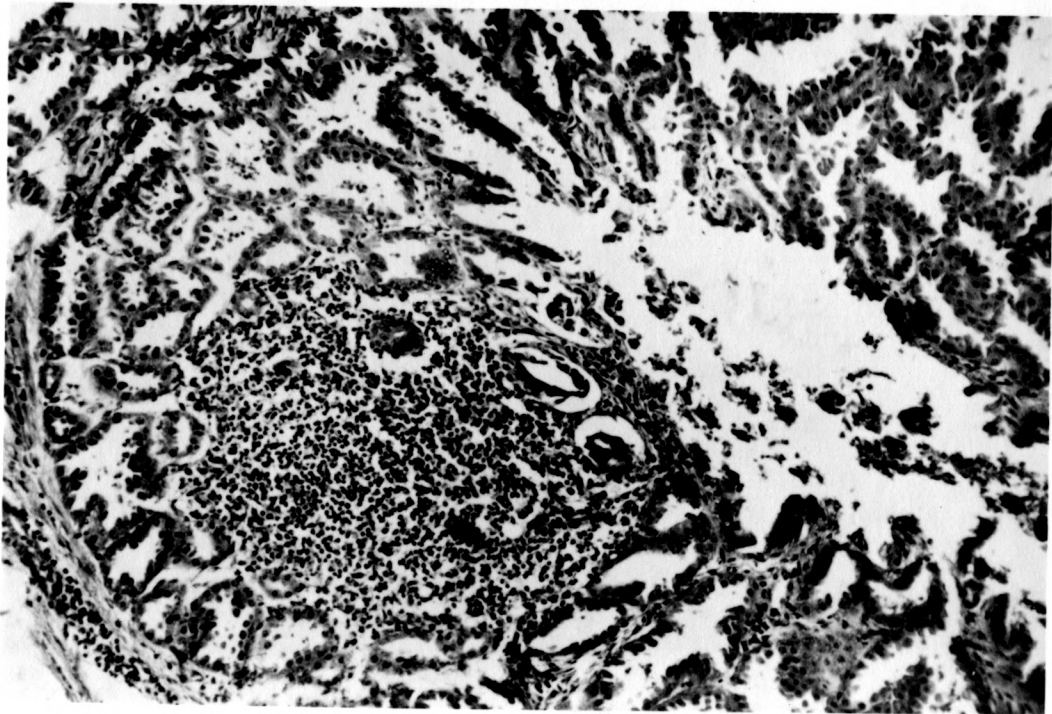
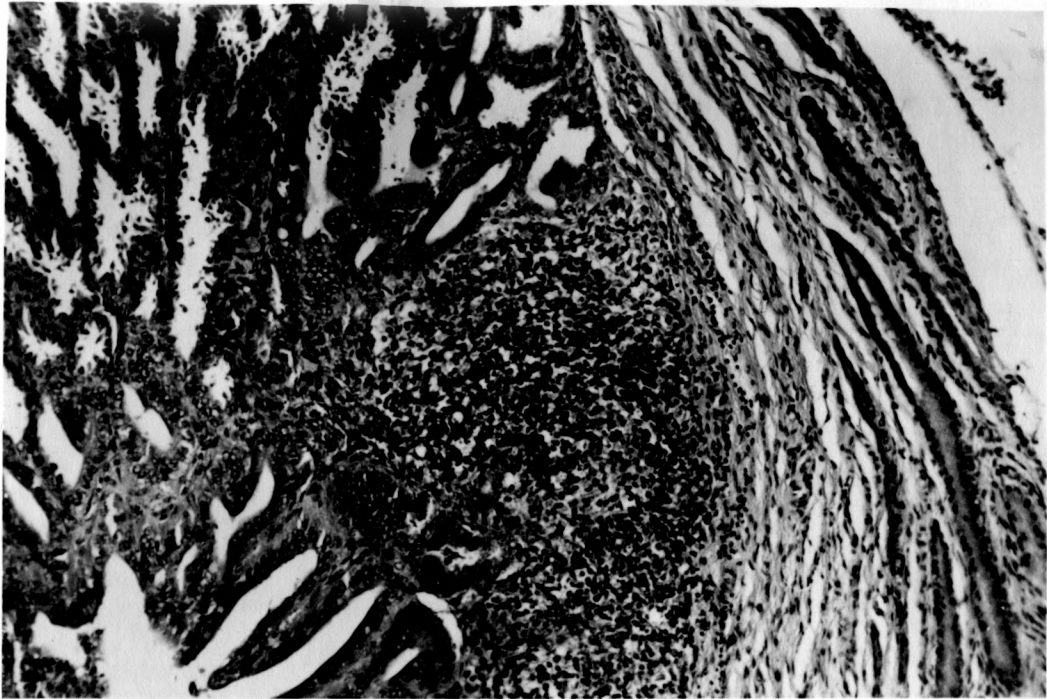


Plate IV

Figure 7. Cerebellum. Perivascular cuffing in granular layer and focal areas of lymphocytic infiltration in molecular layer. H E, X 100.

Figure 8. Cerebellum. Diffuse and focal lymphocytic infiltration of molecular layer, H E, X 100.

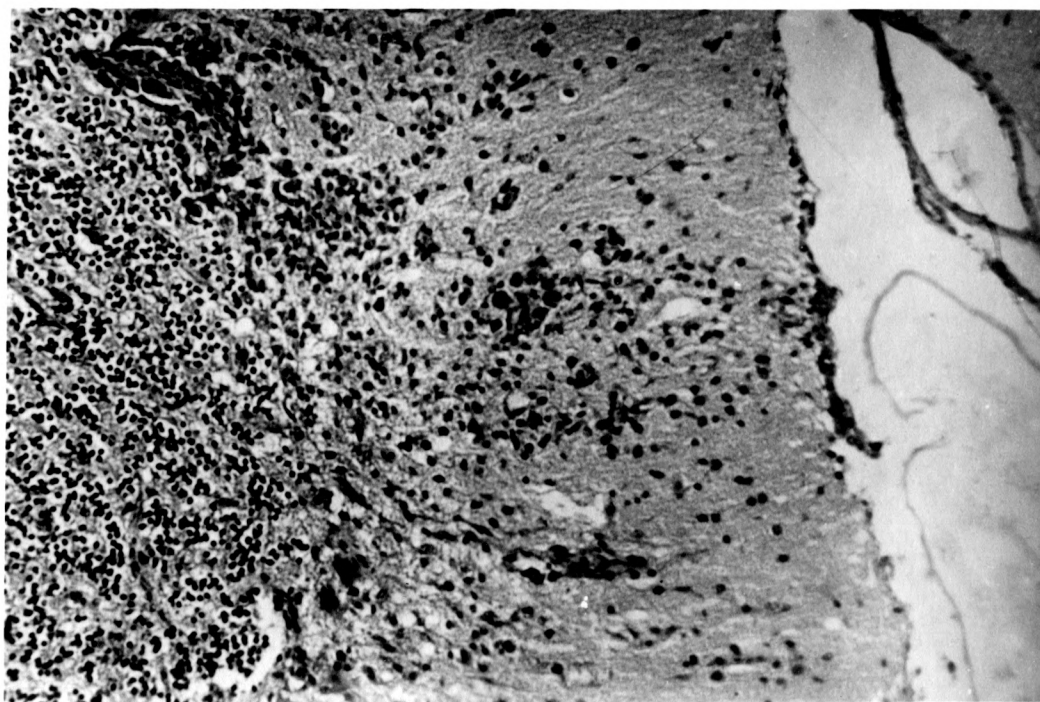
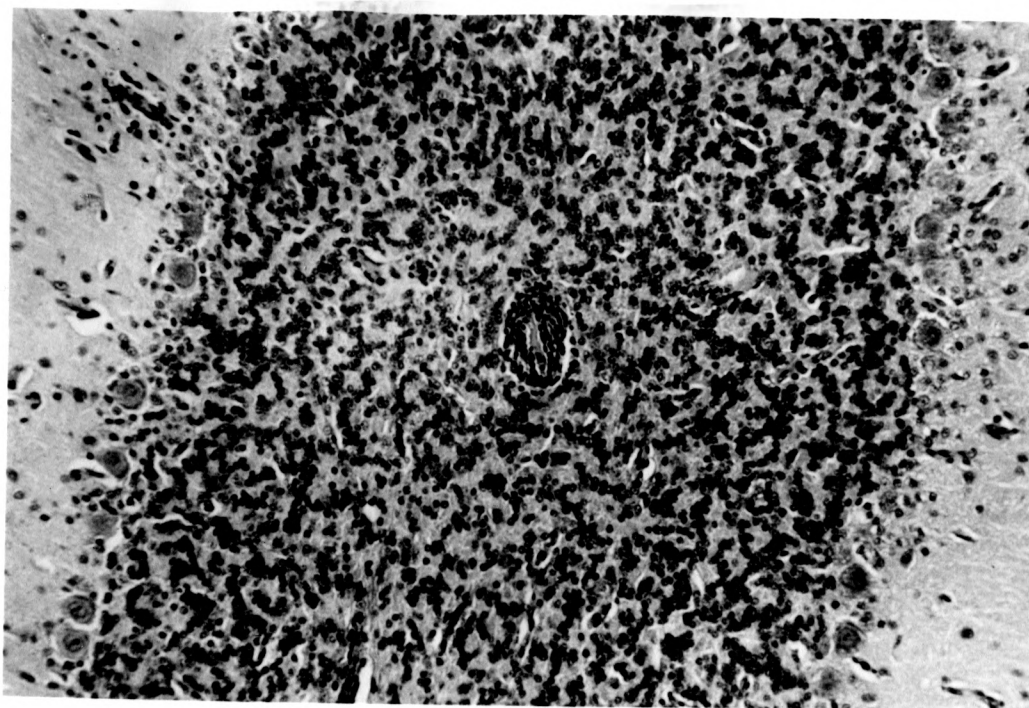


Plate V

Figure 9. Lung. Characteristic fluorescence associated with bronchial epithelium and inflammatory cellular debris in bronchial lumen. FAT, X 100.

Figure 10. Trachea. Fluorescence associated with tracheal epithelium and macrophages in tracheal lumen. FAT, X 100.

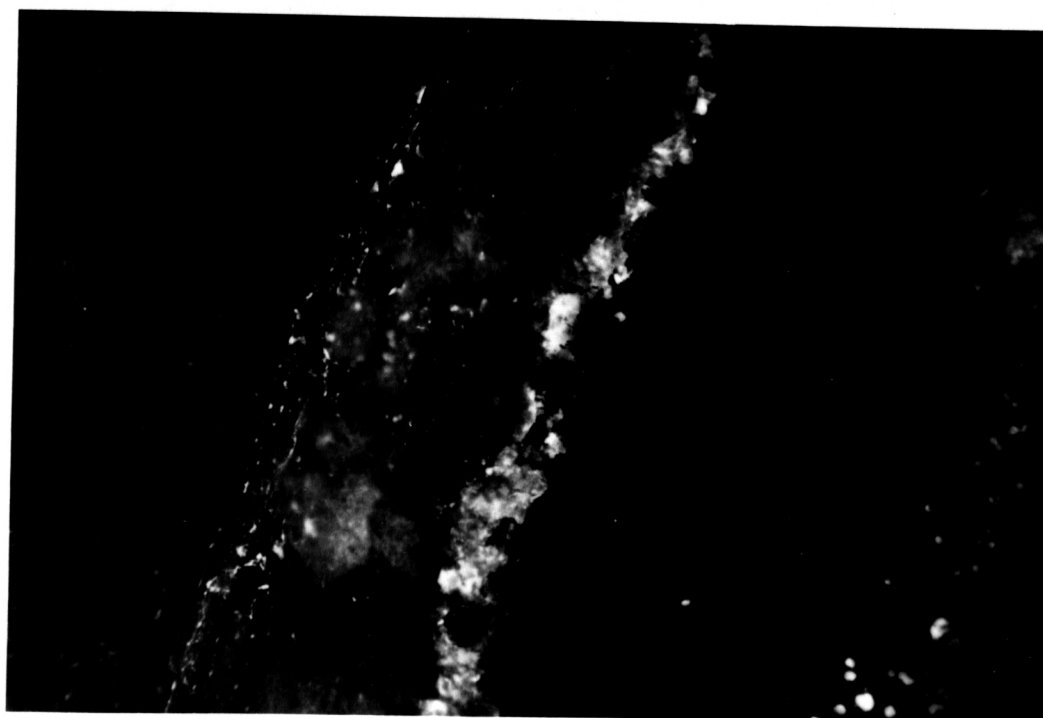
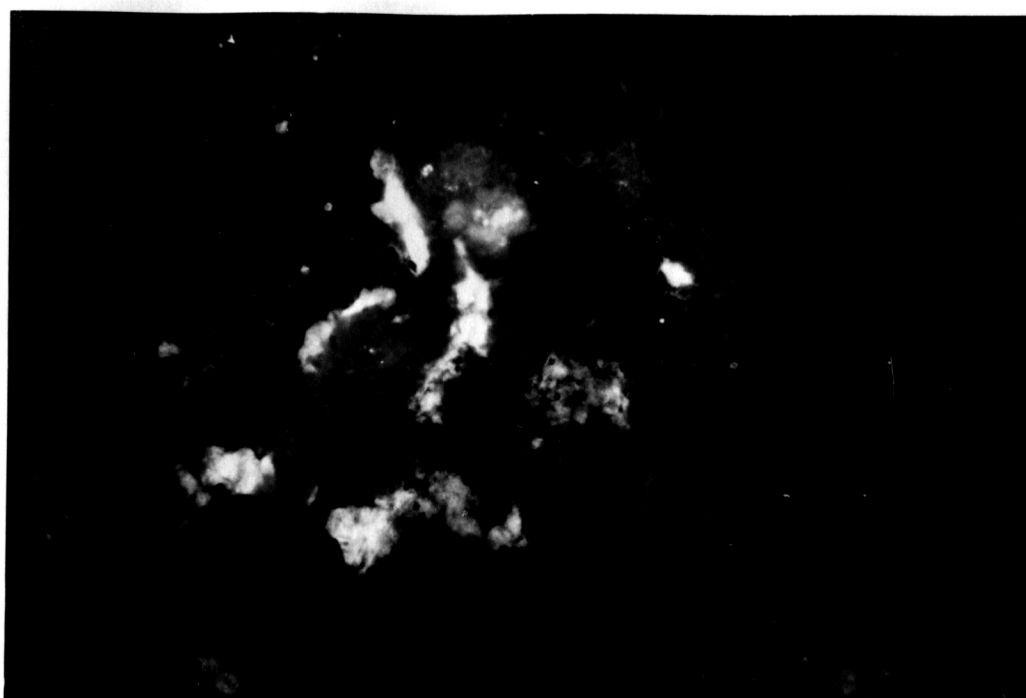
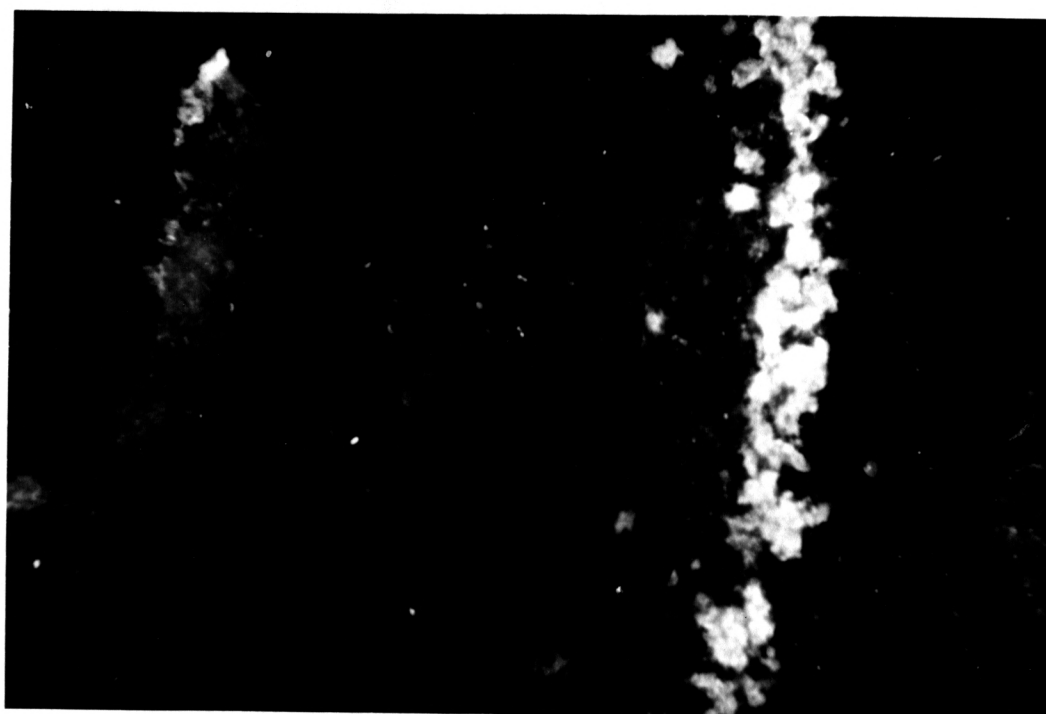
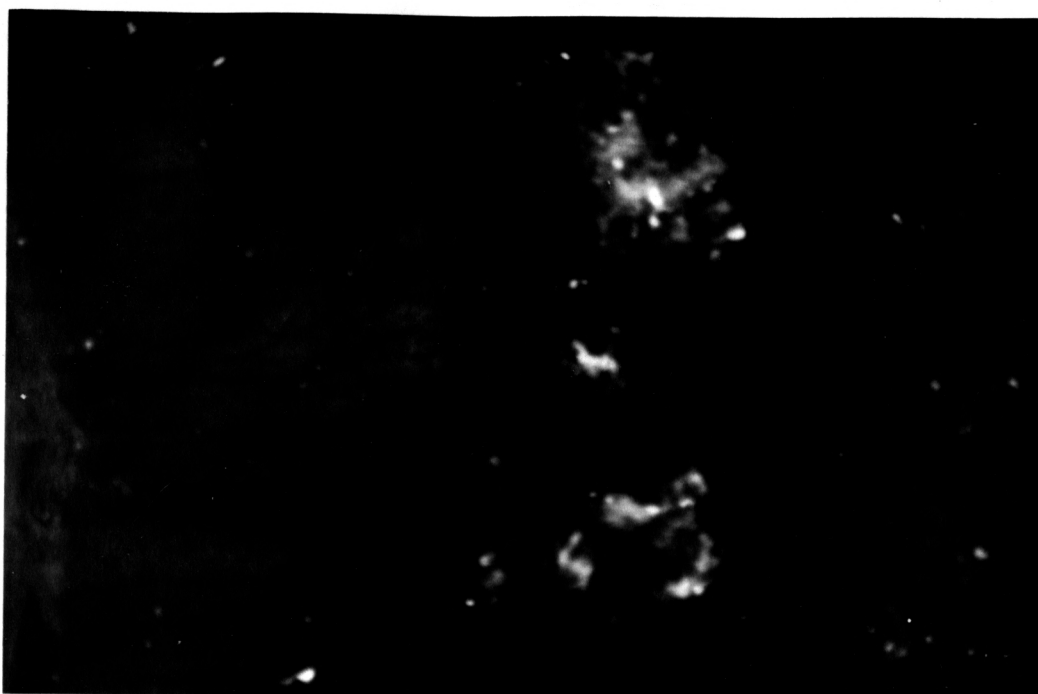


Plate VI

Figure 11. Proventriculus. Fluorescence associated with microulcers in parietal glands. FAT, X 100.

Figure 12. Trachea. Fluorescence associated with tracheal epithelium. FAT, X 250.



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APPENDICES

Appendix A

Results of EI, FAT, and Histopathologic examination for tissues of 5-week old chickens inoculated intramuscularly with 0.1 ml of stock (AF) dilutions (10^{-5} , 10^{-6} , 10^{-7} , 10^{-8}) of NDV.

CHICKEN NUMBER	DAY OF DEATH (CHICKEN)	LUNG			PROVENT			TRACHEA			SPLEEN			BRAIN			BONE MARROW		INOCULUM (AF) DILUTION
		EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT	
1	4th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+	10^{-5}
2		+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+	10^{-5}
3		+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	10^{-6}
4		+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+	10^{-6}
5		+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	10^{-6}
6		+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+	10^{-8}
7	5th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	+	+	+	10^{-5}
8		+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+	10^{-6}
9		+	+	+	+	+	+	+	+	+	+	+	+	0	0	+	+	+	10^{-7}
10	6th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+	10^{-7}
11		+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	10^{-7}
12		+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	0	10^{-8}
13	7th	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	0	10^{-5}
14	12th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	0	10^{-5}
15		+	+	+	+	+	+	+	+	0	+	0	+	0	0	0	+	0	10^{-6}
16		+	0	0	0	0	0	+	0	0	+	0	+	0	0	0	0	0	10^{-7}
17		+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+	10^{-7}
18		+	+	0	+	+	+	+	+	0	+	+	+	+	+	+	+	0	10^{-8}
19		+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	10^{-8}
20		+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	+	0	10^{-8}

AF = Allantoic Fluid

+

0 = negative

EI = Embryo inoculation

HP = Histopathology

FAT = Fluorescent Antibody Technique

Appendix B

Results of EI, FAT and Histopathologic examination for tissues of 7-week old chickens inoculated intramuscularly with $100 \times 10^{8.3}$ ELD₅₀/ml of stock AF NDV.

CHICKEN NUMBER	DAY OF DEATH (CHICKEN)	LUNG			PROVENT.			TRACHEA			SPLEEN			BRAIN			BONE MARROW	
		EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT
1	2nd	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	0
2	"	+	+	+	+	+	+	+	0	+	+	+	+	0	0	0	+	+
3	4th	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+
4	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+
5	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+
6	5th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0
7	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	+	+	0
8	6th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0
9	"	+	+	+	+	+	+	+	+	+	+	0	+	0	0	+	0	0
10	8th	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
11	"	+	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	0
12	10th	+	+	+	+	+	+	+	+	+	+	0	+	0	0	0	0	0
13	"	+	+	+	+	+	0	+	+	+	0	0	0	0	0	0	0	0
14	12th	+	+	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0
15	"	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0

+ = positive

0 = negative

EI = Embryo inoculation

HP = Histopathology

FAT = Fluorescent Antibody Technique

Appendix C

Results of EI, FAT and Histopathologic examination for tissues of 9-week old chickens inoculated intramuscularly with $100 \times 10^{8.3} \text{ELD}_{50}/\text{ml}$ of stock (AF) NDV

CHICKEN NUMBER	DAY OF DEATH (CHICKEN)	LUNG			PROVENT.			TRACHEA			SPLEEN			BRAIN			BONE MARROW	
		EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT	HP	EI	FAT
1	2nd	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+
2	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+
3	4th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+
4	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	0
5	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	0
6	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	0
7	5th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	0
8	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	+	+	+
9	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+
10	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	+	+	0
11	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	0
12	6th	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0
13	"	+	+	+	+	+	0	+	+	+	+	0	+	0	0	0	0	0
14	"	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	0
15	8th	+	+	+	+	+	+	+	+	0	+	+	+	0	0	+	0	0
16	"	+	+	+	+	+	+	0	0	0	+	+	0	0	0	0	0	0
17	10th	+	+	+	+	+	+	+	+	+	+	0	+	0	0	0	0	0
18	"	+	+	+	+	+	+	+	+	+	+	0	0	0	0	+	0	0
19	12th	+	0	0	+	+	0	0	0	0	0	0	0	0	0	0	0	0
20	"	+	+	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0

+ = positive

0 = negative

EI = Embryo inoculation

HP = Histopathology

FAT = Fluorescent Antibody Technique

COMPARATIVE STUDIES IN THE DIAGNOSIS OF NEWCASTLE
DISEASE, IN EXPERIMENTALLY INFECTED CHICKENS,
USING EGG INOCULATION, FLUORESCENT ANTIBODY
AND HISTOPATHOLOGIC TECHNIQUES

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Three groups of White Leghorn chickens (5, 7, and 9 weeks old) were inoculated, intramuscularly, with various dilutions of Newcastle disease virus (NDV), Salsbury strain. During 12-day periods post inoculation, chickens with severe clinical signs were euthanatized, by exsanguination, and necropsied. Lung, trachea, proventriculus, brain, spleen, and bone marrow were collected. Each tissue was divided into three parts and prepared, by standard methods, for: 1) embryo inoculation and viral isolation in allantoic sacs of 10-day embryonating chicken eggs (EI), 2) detecting viral antigens in the tissues by direct fluorescent antibody technique (FAT), and 3) microscopic examination of tissues for lesions caused by the virus.

Higher dosages of the virus produced severer clinical signs and lesions, faster embryo mortalities with higher hemagglutinating titers of the allantoic fluid, and brighter, characteristic, tissue associated fluorescence.

The 275 tissues examined by all techniques yielded these positive results: 213 by embryo inoculation, 204 by microscopic examination, and 195 by direct fluorescent antibody technique.

Histologic lesions were nonspecific and further comparisons between the results of EI and FAT showed 90 percent agreement. All diagnostic tests showed some evidence of NDV infection, in various combinations of tissues, by all infected chickens. Lung, proventriculus, trachea, and spleen were the tissues most frequently infected.

Direct FAT proved the simplest, fastest, and most specific test for routine diagnosis of Newcastle disease (ND) in chickens.